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(54) Title: MATRIX METALLOPROTEINASE PROENZYME ACTIVATOR

(57) Abstract

An MMP–proenzyme activator (MMP–PA) that is expressed by articular cartilage. An MP–PA, biologically active fragments and
mutants thereof, the cleavage products generated by the enzyme, and compounds (e.g., antibodies or small molecules) that modulate the
MMP–PA's activity can be used to diagnose, monitor, and treat conditions in which articular cartilage is either lost or overproduced.

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MATRIX METALLOPROTEINASE PROENZYME ACTIVATOR

Statement as to Federally Sponsored Research

Background of the Invention

          Osteoarthritis is a common and debilitating
10 disease that primarily afflicts the elderly. Its most
prominent feature is the loss of articular cartilage, the
weight-bearing, wear-resistant tissue that provides near
frictionless articulation within healthy synovial joints.
Proteases secreted by the cartilage are believed to
15 contribute to the loss of extracellular matrix in
osteoarthritis. Many of these proteases belong to the
matrix metalloproteinase (MMP) family.
          Most MMPs are secreted as inactive zymogens,
requiring proteolytic release of a conserved propeptide
20 domain for activation. Increased levels of one such
protease, MMP-3, has been detected in cartilage or
cartilage explant culture medium obtained from patients
who have osteoarthritis. MMP-3 is also known as active
neutral metalloproteinase, stromelysin, proteoglycanase,
25 and collagenase activator protein.

Summary of the Invention

          The invention is based on the isolation of a
MMP-proenzyme activator (MMP-PA) that is expressed by
articular cartilage (additional characteristics of this
30 novel polypeptide are described below). This enzyme,
biologically active fragments and mutants thereof, the
cleavage products generated by the enzyme, and compounds
(e.g., antibodies or small molecules) that modulate the
enzyme's activity can be used to diagnose, monitor, and

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treat conditions in which articular cartilage is either
.lost or overproduced.

Accordingly, the invention features an isolated
MMP-PA polypeptide that: (a) activates a matrix
5 metalloproteinase proenzyme (MMP); (b) is produced by
articular cartilage; (c) lacks caseinase and gelatinase
activity; (d) does not degrade bovine serum albumin (BSA)
or plasminogen; and (e) is inhibited by 4-(2-aminoethyl)-
benzenesulfonylfluoride (AEBSF) or benzamidine. The
10 polypeptide is approximately 60-70 kDa in size, is stable
at 55°C, and inactivated at 70°C.

The polypeptide can activate a number of MMPs,
including MMP-3 and MMP-9 (which is also known as 92-k
gelatinase). An MMP-PA of the invention activates a MMP
15 by initiating the removal of some or all of the
propeptide domain of the MMP. Activation can be observed
and quantitated by assays, such as those described herein
(see, e.g., the Examples below and Figs. 2A and 2B), in
which the activity of an MMP is recorded following
20 incubation with a particular concentration of MMP-PA for
a particular time, or recorded over time as a percentage
of the activity obtained following activation of a MMP by
APMA. After incubation of the MMP zymogen with MMP-PA, a
marked increase in the cleavage of labeled casein is
25 evident. Thus, MMP-PAs activate a metalloproteinase
(e.g., a MMP) if, following incubation with the
metalloproteinase, the MMP-PA increases the ability of
the metalloproteinase to cleave casein (or an appropriate
substrate (which would be known to one of skill in the
30 art, e.g., gelatin would be an appropriate substrate in
the event the MMP is MMP-2 or MMP-9)) by at least about
10%, e.g., by 25%, 50%, 100%, or more (relative to the
ability of the inactivated metalloproteinase (i.e.,
metalloproteinase untreated with MMP-PA) to cleave
35 casein).

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An MMP-PA of the invention lacks caseinase and
gelatinase activity (when analyzed, *e.g.*, on substrate
gels prepared and run essentially as described by Heussen
et al. (*Anal. Biochem.* 102:196-202, 1980; see also the
5 "Methods" section of the Examples and Towle *et al.*,
Biochem. Biophys. Res. Commun. 247:324-331, 1998).

An MMP-PA of the invention can lack the ability to
degrade bovine serum albumin (BSA) or plasminogen. The
electrophoretic mobilities of BSA and plasminogen can be
10 assessed, *e.g.*, after incubation under conditions that
completely convert proMMP-3 to an active form (as
described further below). Degradation (or digestion) of
BSA and/or plasminogen can be assessed by SDS gel
electrophoresis (as in the Examples, below; see also
15 Towle *et al.*, *Biochem. Biophys. Res. Commun.* 247:324-331,
1998). BSA and/or plasminogen will be at least 50%, more
preferably 75%, and most preferably essentially 100%
intact (*i.e.*, undegraded) following incubation with an
MMP-PA of the invention under the conditions described
20 below (*i.e.*, incubation in 15 µl B-50 with 22 ng of a
MMP-PA for 18 hours at 37°C).

An MMP-PA of the invention is stable at 55°C and
inactive at 70°C. That is, preincubating the MMP-PA at
55°C for 20 minutes decreases subsequent MMP activation
25 less than 90% (relative to the same MMP-PA preincubated
at 37°C for 20 minutes. Similarly, preincubating the
MMP-PA at 70°C for 20 minutes decreases subsequent MMP
activation more than 50% (e.g., 70% 80% or even more than
90%).

30   An MMP-PA of the invention can be inhibited by 4-
(2-aminoethyl)-benzenesulfonylfluoride (AEBSF) or
benzamidine. To assess inhibition, an MMP can be
incubated with an MMP-PA and any given additive (which
may inhibit, stimulate, or have no effect on the MMP-PA's
35 activity). Active MMP can then be measured using [^{14}C]-

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casein as a substrate (for more specific parameters
useful in carrying out such an assay, see the Examples
below and Table 3). While proMMP-3 activation by MMP-PA
is inhibited by 4-(2-aminoethyl)-benzenesulfonyl-fluoride
5   (AEBSF) and benzamidine, the enzyme was not effectively
inhibited by any other serine proteinase inhibitor tested
(see Table 3).

The MMP-PA of the invention can be administered to
an individual to treat a condition characterized by
10 undesirable growth of tissue (e.g., undesirable bone or
cartilage formation as occurs, for example, in benign
tumors). Alternatively, compounds that inhibit MMP-PA
expression or activity can be identified and administered
to an individual to treat a condition characterized by
15 undesirable bone or cartilage degradation (e.g., an
arthritic condition such as osteoarthritis or rheumatoid
arthritis). Inhibiting MMP-PA activity can also be
useful in treating a patient who has a tumor, as
preventing degradation of the extracellular matrix (ECM)
20 can prevent tumor cells from migrating to other sites
(i.e., metastasis).

In another embodiment, the invention features
antibodies that specifically bind MMP-PAs. As described
further below, these antibodies can be monoclonal or
25 polyclonal antibodies and can inhibit MMP activation by
MMP-PAs. Inhibition of enzyme activity can be assessed
in numerous ways, which will be readily apparent to one
of ordinary skill in the art. For example, one can
assess MMP-PA activity directly (e.g., by assessing the
30 ability of an MMP-PA to initiate cleavage of a MMP, e.g.,
MMP-3 or MMP-9). Alternatively, one can assess MMP-PA
activity indirectly, i.e., by assessing the activity of
an MMP upon which it acts. For example, one can assess
MMP-PAs by assessing the ability of MMP-3 to cleave
35 casein. An MMP-PA of the invention (e.g., a fragment or

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other mutant of the full length MMP-PA described herein)
will initiate cleavage of an MMP (e.g., MMP-3 or MMP-9)
so that that MMP degrades casein at least about 10%,
e.g., 25%, 50%, or 100% more than it did prior to
5   activation by the MMP-PA. Also within the invention are
antibodies (e.g., monoclonal antibodies) that recognize
MMP neoepitopes.

        The substances described herein, including agents
that stimulate or inhibit MMP-PA expression or activity
10   (e.g., anti-MMP-PA antibodies) and MMP-PA polypeptides,
or biologically active portions thereof (which can be
identified by any assay, such as those described herein,
for assessing the activity of the full length MMP-PA),
can be incorporated into pharmaceutical compositions
15   that, optionally, include pharmaceutically acceptable
carriers.

        Another aspect of the invention provides methods
for detecting MMP-PA expression or activity in a
biological sample (e.g., a sample of blood, cartilage, or
20   synovial fluid). The method can be carried out, for
example, by contacting the biological sample with a
nucleic acid probe that hybridizes with MMP-PA MRNA, an
anti-MMP-PA antibody, or an agent that, directly or
indirectly, detects MMP-PA activity (e.g., an agent that
25   detects activation of MMP-3 or MMP-9, e.g., by detecting
the products that are formed when one of these MMPs is
converted from an inactive to an active form).
Biological samples include samples of tissues, cells, or
fluids isolated from a subject, as well as tissues,
30   cells, or fluids present within a subject. Thus, the
detection methods of the invention (whether employed to
diagnose a patient, treat a patient, monitor a clinical
trial, etc.) can be used to analyze a biological sample
in vitro or in vivo. Preferably, the biological sample

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is a sample of cartilage, synovial fluid, or blood, which
can be obtained from a subject by conventional means.

Thus, methods in which MMP-PAs, or the products
formed when these proteinases are active, are detected
5 can be practiced to make or confirm a medical diagnosis
(e.g., that a patient has osteoarthritis or any other
condition in which articular cartilage is lost) or to
monitor the progression of articular cartilage loss
(e.g., during a clinical trial or treatment regime). For
10 example, in a clinical trial of an agent that is designed
to prevent, slow, or halt the loss of articular cartilage
(by, e.g., inhibiting an MMP-PA of the invention), the
expression or activity of the MMP-PA can be assessed in
groups of treated and untreated patients and correlated
15 with the loss of articular cartilage.

Another aspect of the invention features a method
for modulating MMP-PA activity. The method can be
carried out, for example, by contacting a cell with an
agent that modulates (i.e., inhibits or stimulates) MMP-
20 PA expression or activity. Preferably, MMP-PA expression
or activity is modulated in vivo. The modulatory agent
can be an antibody that specifically binds to MMP-PA or
an agent that modulates transcription of a MMP-PA gene,
splicing of a MMP-PA mRNA, or translation of a MMP-PA
25 mRNA.

In another aspect, the invention provides a
method for identifying a compound that binds to or
modulates the activity of a MMP-PA protein. In general,
such methods entail measuring a biological activity of
30 MMP-PA in the presence and absence of a test compound and
identifying those compounds which alter the activity of
MMP-PA.

Similarly, the invention features methods for
identifying a compound that modulates the expression of
35 MMP-PA by measuring the expression of MMP-PA in the

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presence and absence of a compound. A change in
expression in the presence of the compound, relative to
that seen in the absence of the compound, indicates that
the compound modulates the expression of MMP-PA.

5   In one embodiment, the methods of the present
invention are used to treat a subject having a disorder
characterized by aberrant MMP-PA activity (e.g., an
arthritic condition, such as osteoarthritis) by
administering an agent that is a MMP-PA modulator to the

10   subject. Where the patient requires stimulation of
MMP-PA activity, the MMP-PA modulator can be a MMP-PA
protein. The MMP-PA modulator can also be a peptide,
peptidomimetic, or other small molecule.

          Although methods and materials similar or

15   equivalent to those described herein can be used in the
practice or testing of the present invention, the
preferred methods and materials are described below. All
publications, patent applications, patents, and other
references mentioned herein are incorporated by reference

20   in their entirety.

          Other features and advantages of the invention
will be apparent from the following detailed description,
and from the claims.

                 Brief Description of the Drawings

25   Fig. 1A is a gel filtration chromatograph
(Sephacryl S-200) of MMP-PA. Active material (400 μg)
obtained from the Matrex Gel Green step was
chromatographed on a Sephacryl S-200 column equilibrated
in B-50. Two ml fractions were collected and monitored

30   for protein (estimated from absorbance at 280 nM).
Aliquots of column fractions (0.5 μl of 2 ml) were tested
for the capacity to activate bovine proMMP-3 using [^{14}C]-
casein as a substrate for active MMP-3.   Fig. 1B is a
photograph of an SDS-polyacrylamide gel. Proteins in

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fractions (20 μl of 2 ml) corresponding to the major peak
of activity (Fig. 1A) were analyzed by SDS-PAGE under
reducing conditions. Protein standards (bovine serum
albumin, ovalbumin and carbonic anhydrase; Rainbow
5 protein standards, Amersham) are indicated by bars at
left.   Fig. 1C is a photograph of an SDS-
polyacrylamide gel. Aliquots (5 μl) of each fraction
shown in Fig. 1B were tested for the capacity to cleave
300 ng bovine proMMP-3 in the presence of excess TIMP-1,
10 with digestion products resolved on an SDS polyacrylamide
gel. The proenzyme form of MMP-3 is indicated by arrows.
The gels were stained with silver.
        Figs. 2A and 2B are line graphs indicating
activation of human fibroblast proMMP-3 by MMP-PA.   In
15 Fig. 2A, human proMMP-3 (37.6 μg/ml) was incubated for
4 hours with various amounts of MMP-PA in 10 μl B-50.
Subsequent to the activating incubation, active MMP-3 in
3 μl of each reaction product was measured using labeled
casein as a substrate. Data are expressed as a
20 percentage of the casein degraded by APMA-activated MMP-
3. Casein digestion by MMP-PA (2.2 μg/ml for 4 hours)
was undetectable in this assay. In Fig. 2B, human
proMMP-3 (33 μg/ml) was incubated for various times
without exogenous activator (open circles) or with 1.1
25 μg/ml MMP-PA (filled circles) in 10 μl B-50. Active MMP-
3 in 2 μl of each reaction product was measured using the
labeled casein substrate, with activity expressed as a
percentage of that obtained following activation by APMA.
        Fig. 3 is a photograph of an SDS-polyacrylamide
30 gel illustrating bovine proMMP-3 activation intermediates
generated by MMP-PA. Bovine proMMP-3 (1 μg) was
incubated in 20 μl B-50 without exogenous activator (lane
2), with 1 mM APMA (lane 3), or with various amounts of
MMP-PA (11 ng, 22 ng, or 44 ng, lanes 4-6) in the
35 presence of human recombinant TIMP-1. Reaction products

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were resolved by SDS-PAGE, and the gels were stained with
Coomassie Blue R250. Lane 1 contains proMMP-3 without
incubation, and arrows indicate the MMP-3 activation
products that accumulated during incubation with MMP-PA.

5   Fig. 4 is a diagrammatic representation of proMMP-
3, which includes a propeptide (SEQ ID NO:__), a central
catalytic domain, and a C-terminal hemopexin domain.
Removal of some or all of the propeptide domain activates
the enzyme. MMP-PA is predicted to hydrolyze the
10 methionine$_{70}$-arginine$_{71}$ and arginine$_{71}$-lysine$_{72}$ peptide
bonds in the human MMP-3 propeptide based on the
alignment of sequences of bovine MMP-3 activation
intermediates generated by this enzyme. At least one
larger Mr activation intermediate was transiently present
15 during incubation of proMMP-3 with MMP-PA, but it did not
accumulate to levels sufficient for sequence analysis.
The boxed region in the human MMP-3 propeptide is
susceptible to a variety of proteinases, and subsequent
MMP-3-dependent cleavage at the site indicated by the
20 arrow generates a unique active species with a
phenylalanine amino terminus.

                        Detailed Description
        The present invention is based on the isolation of
an activator of metalloproteinases (e.g., matrix
25 metalloproteinases).
        I. Isolated MMP-PA Proteins and Anti-MMP-PA Antibodies
                Isolated MMP-PAs, biologically active portions
thereof, and polypeptide fragments can be used as
immunogens to generate anti-MMP-PA antibodies. Native
30 MMP-PAs can be isolated from cells or tissues by an
appropriate purification scheme using standard protein
purification techniques. MMP-PAs can also be
recombinantly produced or chemically synthesized using

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techniques routinely practiced by those of ordinary skill
in the art.

As used herein, the term "polypeptide" refers to a
chain of at least two amino acid residues, which may or
5 may not be modified (e.g., glycosylated). The terms
"polypeptide," "peptide," and "protein" are considered
equivalent and may be used interchangeably herein. An
"isolated" or "purified" polypeptide is substantially
free of contaminating proteins or other cellular
10 materials associated with the cell or tissue from which
the polypeptide (e.g., an MMP-PA) was isolated (or in
which the polypeptide was recombinantly produced) or from
chemical precursors or other substances used in the
process of chemically synthesizing the polypeptide.
15 Preparations of MMP-PAs that are "isolated" or "purified"
are preparations of MMP-PAs that have less than about
30%, 20%, 10%, or 5% (by dry weight) of non-MMP-PA
protein (also referred to herein as a "contaminating
protein"). When an MMP-PA, or a fragment (e.g., a
20 biologically active portion) thereof is recombinantly
produced, it is preferably substantially free of culture
medium, i.e., culture medium represents less than about
30%, 20%, 10%, or 5% of the volume of the protein
preparation.
25   As stated above, an isolated MMP-PA, or a portion
or fragment thereof, can be used as an immunogen to
generate antibodies that bind MMP-PA. These antibodies
can be generated using standard techniques for preparing
polyclonal and monoclonal antibodies.
30   More specifically, MMP-PA immunogens are typically
used to prepare antibodies by immunizing a suitable
subject (e.g., a rabbit, goat, mouse, or other mammal)
with the immunogen. An appropriate immunogenic
preparation can contain, for example, purified MMP-PAs,
35 recombinantly expressed MMP-PAs, or chemically

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synthesized MMP-PAs. The preparation can also include an
adjuvant, such as Freund's complete or incomplete
adjuvant, or a similar immunostimulatory agent.
Immunization of a suitable subject with an immunogenic
5 MMP-PA preparation induces a polyclonal anti-MMP-PA
antibody response.

The term "antibody" as used herein refers to
immunoglobulin molecules and immunologically active
portions of immunoglobulin molecules, i.e., molecules
10 that contain an antigen binding site that specifically
binds an antigen (e.g., an MMP-PA). A molecule that
specifically binds to an MMP-PA is a molecule that binds
an MMP-PA, but does not substantially bind other
molecules in a sample, for example, a biological sample,
15 which contains an MMP-PA. Examples of immunologically
active portions of immunoglobulin molecules include F(ab)
and F(ab')$_2$ fragments which can be generated by treating
an antibody with an enzyme such as pepsin. The invention
provides polyclonal and monoclonal antibodies that bind
20 an MMP-PA. The terms "monoclonal antibody" and
"monoclonal antibody composition," as used herein, refer
to a population of antibody molecules that contain only
one species of an antigen binding site capable of
immunoreacting with a particular epitope of MMP-PA. A
25 monoclonal antibody composition thus typically displays a
single binding affinity for a particular MMP-PA with
which it immunoreacts.

Antibodies that specifically bind to MMP
neoepitopes (newly created epitopes having new amino and
30 carboxy terminal ends, which are generated when a MMP is
cleaved) are also within the scope of the invention.
Antibodies raised against synthetic peptides
corresponding to these epitopes can be used to identify
or detect the cleaved MMP products in a biological fluid.
35 More specifically, antibodies can be generated against

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the following peptides: KDVKQFV (SEQ ID NO:1), RRKDSGP
(SEQ ID NO:2), SDTLEVM (SEQ ID NO:3), RKPRCGV (SEQ ID
NO:4), GYTRVAEM (SEQ ID NO:5), RGESKSL (SEQ ID NO:6),
SATLKAM (SEQ ID NO:7), and RTPRCGV (SEQ ID NO:8). Any of
5 these polypeptide immunogens (as well as others disclosed
herein) can be linked to keyhole limpet hemocyanin.
      Polyclonal anti-MMP-PA antibodies can be prepared
as described above by immunizing a suitable subject with
a MMP-PA immunogen. The anti-MMP-PA antibody titer in
10 the immunized subject can be monitored over time by
standard techniques, such as with an enzyme linked
immunosorbent assay (ELISA) using immobilized MMP-PA. If
desired, the antibody molecules directed against MMP-PA
can be isolated from the mammal (e.g., from the blood)
15 and further purified by well-known techniques, such as
protein A chromatography to obtain the IgG fraction. At
an appropriate time after immunization, for example, when
the anti-MMP-PA antibody titers are highest, antibody-
producing cells can be obtained from the subject and used
20 to prepare monoclonal antibodies by standard techniques,
such as the hybridoma technique originally described by
Kohler and Milstein (Nature 256:495-497, 1975), the human
B cell hybridoma technique (Kozbor et al., Immunol. Today
4:72, 1983), the EBV-hybridoma technique (Cole et al.,
25 Monoclonal Antibodies and Cancer Therapy, Alan R. Liss,
Inc., pp. 77-96, 1985) or trioma techniques. The
technology for producing various monoclonal antibody
hybridomas is well known (see, e.g., Coligan et al.
(eds.) Current Protocols in Immunology John Wiley & Sons,
30 Inc., New York, NY, 1994). Briefly, an immortal cell
line (typically a myeloma) is fused to lymphocytes
(typically splenocytes) from a mammal immunized with a
MMP-PA immunogen as described above, and the culture
supernatants of the resulting hybridoma cells are

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screened to identify a hybridoma producing a monoclonal
antibody that binds MMP-PA.

Any of the many well known protocols used for
fusing lymphocytes and immortalized cell lines can be
5 applied for the purpose of generating an anti-MMP-PA
monoclonal antibody (see, *e.g.*, Current Protocols in
Immunology, *supra*; Galfre *et al.*, *Nature* 266:55052, 1977;
R.H. Kenneth, in *Monoclonal Antibodies: A New Dimension
In Biological Analyses*, Plenum Publishing Corp., New
10 York, NY, 1980; and Lerner *Yale J. Biol. Med.* 54:387-402,
1981). Moreover, one of ordinary skill in the art will
appreciate that there are many variations of such methods
which would also be useful in the context of the present
invention. Typically, the immortal cell line (*e.g.*, a
15 myeloma cell line) is derived from the same mammalian
species as the lymphocytes. For example, murine
hybridomas can be made by fusing lymphocytes from a mouse
immunized with an immunogenic preparation of MMP-PA with
an immortalized mouse cell line, for example, a myeloma
20 cell line that is sensitive to culture medium containing
hypoxanthine, aminopterin, and thymidine ("HAT medium").
Any of a number of myeloma cell lines can be used as a
fusion partner according to standard techniques, for
example, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14
25 myeloma lines. These myeloma lines are available from
the American Type Culture Collection (Manassas, VA).
Typically, HAT-sensitive mouse myeloma cells are fused to
mouse splenocytes using polyethylene glycol (PEG).
Hybridoma cells resulting from the fusion are then
30 selected using HAT medium, which kills unfused and
unproductively fused myeloma cells (unfused splenocytes
die after several days because they are not transformed).
Hybridoma cells producing a monoclonal antibody of the
invention are detected by screening the hybridoma culture

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supernatants for antibodies that bind MMP-PA, for
example, using a standard ELISA assay.

Instead of preparing monoclonal antibody-secreting
hybridomas, a monoclonal anti-MMP-PA antibody can be
5   identified and isolated by screening a recombinant
combinatorial immunoglobulin library (e.g., an antibody
phage display library) with MMP-PA to thereby isolate
members of the immunoglobulin library that bind MMP-PA.
Kits for generating and screening phage display libraries
10   are commercially available (e.g., the Pharmacia
Recombinant Phage Antibody System, Catalog No. 27-9400-
01; and the Stratagene SurfZAP™ Phage Display Kit,
Catalog No. 240612).

An anti-MMP-PA antibody (e.g., a monoclonal
15   antibody) can be used to isolate MMP-PA by standard
techniques, such as affinity chromatography or
immunoprecipitation. An anti-MMP-PA antibody can
facilitate the purification of natural MMP-PA from cells
and of recombinantly produced MMP-PA from host cells.
20   Moreover, an anti-MMP-PA antibody can be used to detect
MMP-PA protein (e.g., in a cellular lysate or cell
supernatant) in order to evaluate the abundance and
pattern of expression of the MMP-PA protein. Anti-MMP-PA
antibodies can be used diagnostically to monitor protein
25   levels in tissue as part of a clinical testing procedure
to, for example, determine the efficacy of a given
treatment regimen. Detection can be facilitated by
coupling the antibody to a detectable substance.
Detectable substances are well known to those of ordinary
30   skill in the art and include various enzymes, prosthetic
groups, fluorescent materials, luminescent materials,
bioluminescent materials, and radioactive materials.

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II.   Pharmaceutical Compositions

        The MMP-PA polypeptides and anti-MMP-PA antibodies
(also referred to herein as "active compounds") of the
invention can be incorporated into pharmaceutical
5   compositions suitable for administration to a subject
(e.g., a human patient). These compositions typically
include the polypeptide or antibody and a
pharmaceutically acceptable carrier. As used herein, the
term "pharmaceutically acceptable carrier" is intended to
10 include any and all solvents, dispersion media, coatings,
antibacterial and antifungal agents, isotonic and
absorption delaying agents, and the like, compatible with
pharmaceutical administration. The use of such media and
agents for pharmaceutically active substances is well
15 known in the art. Use of these carriers in therapeutic
compositions is contemplated, except insofar as they are
incompatible with the active compound (a determination
one of ordinary skill in the art can readily make).
Supplementary active compounds can also be incorporated
20 into the compositions.

        Naturally, a pharmaceutical composition of the
invention will be formulated in a manner compatible with
its intended route of administration. Examples of routes
of administration include oral administration and
25 parenteral administration. For example, a pharmaceutical
composition can be formulated for oral, intravenous (or
intraarterial), intradermal, intramuscular, subcutaneous,
oral, transdermal, or transmucosal (e.g., nasal or
rectal) administration. Similarly, a pharamaceutical
30 composition can be formulated for application to the area
surrounding a joint (e.g., injection into the synovial
fluid). The solutions, suspensions, gels, ointments, or
creams used for administration can include one or more of
the following components: a sterile diluent such as
35 water, saline solution, fixed oils, polyethylene glycols,

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glycerine, propylene glycol, or other synthetic solvents;
antibacterial agents such as benzyl alcohol or methyl
parabens; antioxidants such as ascorbic acid or sodium
bisulfite; chelating agents such as ethylenediamine-
5   tetraacetic acid; buffers such as acetates, citrates, or
phosphates; and agents for the adjustment of tonicity
such as sodium chloride or dextrose. The pH of a
pharmaceutical preparation can be adjusted with acids or
bases, such as hydrochloric acid or sodium hydroxide.
10   Moreover, the preparations can be enclosed in ampoules,
disposable syringes, or multiple dose vials made of glass
or plastic.

Pharmaceutical compositions suitable for injection
include sterile aqueous solutions (where the active
15   ingredient is water soluble) or dispersions and sterile
powders for the extemporaneous preparation of sterile
injectable solutions or dispersion. For intravenous
administration, suitable carriers include physiological
saline, bacteriostatic water, Cremophor EL™ (BASF;
20   Parsippany, NJ) or phosphate buffered saline (PBS). In
all cases, the composition must be sterile and should be
fluid to the extent that easy syringability exists. It
must be stable under the conditions of manufacture and
storage and must be preserved against the contaminating
25   action of microorganisms such as bacteria and fungi. The
carrier can be a solvent or dispersion medium containing,
for example, water, ethanol, polyol (for example,
glycerol, propylene glycol, and liquid polyetheylene
glycol, and the like), and suitable mixtures thereof.
30   The proper fluidity can be maintained, for example, by
the use of a coating such as lecithin, by the maintenance
of the required particle size in the case of dispersion
and by the use of surfactants. Prevention of the action
of microorganisms can be achieved by various
35   antibacterial and antifungal agents, for example,

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parabens, chlorobutanol, phenol, ascorbic acid,
thimerosal, and the like. In many cases, it will be
preferable to include isotonic agents, for example,
sugars, polyalcohols such as mannitol, sorbitol, and
5   sodium chloride in the composition. Prolonged absorption
of the injectable compositions can be brought about by
including in the composition an agent that delays
absorption, for example, aluminum monostearate and
gelatin.

10   Sterile injectable solutions can be prepared by
incorporating the active compound (e.g., MMP-PAs or anti-
MMP-PA antibodies) in the required amount in an
appropriate solvent with one or a combination of
ingredients enumerated above, as required, and
15 sterilizing (e.g., filter sterilizing) the solution.
Generally, dispersions are prepared by incorporating the
active compound into a sterile vehicle that contains a
basic dispersion medium and any other required
ingredients (e.g., ingredients selected from those
20 enumerated above).

Oral compositions generally include an inert
diluent or an edible carrier. They can be enclosed in
gelatin capsules or compressed into tablets. For the
purpose of oral therapeutic administration, the active
25 compound can be incorporated with excipients and used in
the form of tablets, troches, or capsules. Oral
compositions can also be prepared using a fluid carrier
for use as a mouthwash, wherein the compound in the fluid
carrier is applied orally and swished and expectorated or
30 swallowed. Pharmaceutically compatible binding agents
and/or adjuvant materials can be included as part of the
composition. The tablets, pills, capsules, troches, and
the like can contain any of the following ingredients, or
compounds of a similar nature: a binder such as
35 microcrystalline cellulose, gum tragacanth or gelatin; an

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excipient such as starch or lactose, a disintegrating
agent such as alginic acid, Primogel, or corn starch; a
lubricant such as magnesium stearate or Sterotes; a
glidant such as colloidal silicon dioxide; a sweetening
5 agent such as sucrose or saccharin; or a flavoring agent
such as peppermint, methyl salicylate, or orange
flavoring.   Systemic administration can also be by
transmucosal or transdermal means. For transmucosal or
transdermal administration, penetrants appropriate to the
10 barrier to be permeated are used in the formulation.
Such penetrants are generally known in the art, and
include, for example, for transmucosal administration,
detergents, bile salts, and fusidic acid derivatives.
Transmucosal administration can be accomplished through
15 the use of nasal sprays or suppositories. For
transdermal administration, the active compounds are
formulated into ointments, salves, gels, or creams by
methods generally known in the art.
          In one embodiment, the active compounds are
20 prepared with carriers that will protect the compound
against rapid elimination from the body, such as a
controlled release formulation, including implants and
microencapsulated delivery systems. Biodegradable,
biocompatible polymers can be used, such as ethylene
25 vinyl acetate, polyanhydrides, polyglycolic acid,
collagen, polyorthoesters, and polylactic acid. Methods
for preparation of such formulations will be apparent to
those of ordinary skill in the art. The materials can
also be obtained commercially from Alza Corporation and
30 Nova Pharmaceuticals, Inc. Liposomal suspensions can
also be used as pharmaceutically acceptable carriers.
These can be prepared according to methods known to those
of ordinary skill in the art, for example, as described

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in U.S. Patent No. 4,522,811.   It is especially
advantageous to formulate oral or parenteral compositions
in the form of dosage units, as this provides for ease of
administration and uniformity of dosage. A "dosage unit"
5 refers to a physically discrete unit suitable as unitary
dosages for the subject to be treated; each unit
containing a predetermined quantity of active compound
calculated to produce the desired therapeutic effect in
association with the required pharmaceutical carrier.
10 The specification for the dosage unit forms of the
invention are dictated by and directly dependent on the
unique characteristics of the active compound, the
particular therapeutic effect to be achieved, and the
limitations inherent in the art of compounding such an
15 active compound for the treatment of individuals.

III. Uses and Methods of the Invention

        The polypeptides, including antibodies, described
herein can be used in one or more ways. For example,
they can be used in: (a) screening assays,
20 (b) predictive medicine (e.g., in diagnostic assays,
prognostic assays, monitoring clinical trials, and
pharmacogenomics); and (c) methods of treatment (e.g., in
therapeutic and prophylactic regimes). MMP-PAs can
interact with other cellular proteins and can be used to
25 regulate MMP activity, which, in turn, regulates the
degradation of extracellular matrices (e.g., bone or
cartilage). In addition, MMP-PAs can be used to screen
drugs or compounds that modulate MMP-PA expression or
activity, to treat disorders characterized by
30 insufficient or excessive production of MMP-PAs, and to
treat disorders characterized by production of MMP-PAs
that have decreased or aberrant activity compared to the
wild type MMP-PA (e.g., mutant MMP-PAs). In addition,

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anti-MMP-PA antibodies can be used to detect and isolate
MMP-PAs and to modulate their activity.

        The invention also encompasses novel agents
identified by the screening assays described above, and
5   uses for those agents (e.g., in diagnostic and treatment
regimes), as described herein.

Screening Assays

        By practicing a method of the invention (i.e., a
"screening assay") one of ordinary skill in the art can
10 identify modulators, i.e., candidate or test compounds or
agents (e.g., peptides, peptidomimetics, small molecules,
or other drugs) that bind to MMP-PAs or have a
stimulatory or inhibitory effect on, for example, MMP-PA
expression or MMP-PA activity.

15   For example, one can screen candidate or test
compounds that bind to or otherwise modulate the activity
of a MMP-PA polypeptide or a biologically active portion
thereof. The test compounds can be obtained using any of
the numerous libraries known in the art of combinatorial
20 library methods, including: biological libraries;
spatially addressable parallel solid phase or solution
phase libraries; synthetic library methods requiring
deconvolution; the "one-bead one-compound" library
method; and synthetic library methods using affinity
25 chromatography selection. The biological library
approach is limited to peptide libraries, while the other
four approaches are applicable to peptide, non-peptide
oligomer or small molecule libraries of compounds (Lam,
Anticancer Drug Des. 12:145, 1997).

30   Examples of methods for the synthesis of molecular
libraries can be found in the art, for example in:
DeWitt et al., Proc. Natl. Acad. Sci. USA 90:6909, 1993;
Erb et al. Proc. Natl. Acad. Sci. USA 91:11422, 1994;
Zuckermann et al., J. Med. Chem. 37:2678, 1994; Cho

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et al., *Science* 261:1303, 1993; Carrell et al., *Angew.
Chem. Int. Ed. Engl.* 33:2059, 1994; Carell et al., *Angew.
Chem. Int. Ed. Engl.* 33:2061, 1994; and Gallop et al., *J.
Med. Chem.* 37:1233, 1994.

5   Libraries of compounds may be presented in
solution (e.g., Houghten, *Bio/Techniques* 13:412-421,
1992), or on beads (Lam, *Nature* 354:82-84, 1991), chips
(Fodor, *Nature* 364:555-556, 1993), bacteria (U.S. Patent
No. 5,223,409), spores (U.S. Patent Nos. 5,571,698;
10 5,403,484; and 5,223,409), plasmids (Cull et al., *Proc.
Natl. Acad. Sci. USA* 89:1865-1869, 1992) or on phage
(Scott and Smith, *Science* 249:386-390, 1990; Devlin,
Science 249:404-406, 1990; Cwirla et al., *Proc. Natl.
Acad. Sci. USA* 87:6378-6382, 1990; and Felici, *J. Mol.
15 Biol.* 222:301-310, 1991).

An assay to screen candidate or test compounds
that bind to or otherwise modulate the activity of a MMP-
PA polypeptide can be a cell-based assay in which a cell
that expresses a MMP-PA, or a biologically active portion
20 thereof, is contacted with a test compound and the
ability of the test compound to bind to a MMP-PA protein
determined. The cell, for example, can be a yeast cell
or a cell of mammalian origin. Determining the ability
of the test compound to bind to the MMP-PA protein can be
25 accomplished, for example, by coupling the test compound
to a radioisotope or enzymatic label such that binding of
the test compound to the MMP-PA, or biologically active
portion thereof, can be determined by detecting the
labeled compound in a complex. For example, test
30 compounds can be labeled with ^{125}I, ^{35}S, ^{14}C, or ^{3}H, either
directly or indirectly, and the radioisotope can be
detected by direct counting of radioemmission or by
scintillation counting. Alternatively, test compounds
can be enzymatically labeled with, for example,
35 horseradish peroxidase, alkaline phosphatase, or

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luciferase, and the enzymatic label detected by
determination of conversion of an appropriate substrate
to product. The assay can be carried out, for example,
by contacting a cell that expresses an MMP-PA, or a
5 biologically active portion thereof, with a compound
known to bind MMP-PA to form an assay mixture, contacting
the assay mixture with a test compound, and determining
the ability of the test compound to interact with a MMP-
PA protein. One can determine whether the test compound
10 has the ability to interact with a MMP-PA protein by, for
example, determining whether (and optionally, to what.
extent) the test compound has the ability to
preferentially bind to the MMP-PA, or a biologically
active portion thereof, in a manner comparable to that of
15 the known compound.

Alternatively, a cell-based assay can be carried
out by contacting a cell expressing a MMP-PA, or a
biologically active portion thereof, with a test compound
and determining whether (and, optionally, to what extent)
20 the test compound modulates (e.g., stimulates or
inhibits) the activity of the MMP-PA protein (or the
biologically active portion thereof). Determining
whether the test compound modulates the activity of MMP-
PA, or a biologically active portion thereof, can be
25 accomplished, for example, by determining whether the
MMP-PA binds to or otherwise interacts with a MMP-PA
target molecule (i.e., a molecule with which a MMP-PA
binds or interacts in nature, for example, a MMP-PA
substrate (e.g., MMP-3 or MMP-9), a molecule in the
30 extracellular milieu, or a molecule associated with the
external surface of a cell membrane. A MMP-PA target
molecule can be a non-MMP-PA molecule or a MMP-PA the
present invention.

Determining whether the MMP-PA binds to or
35 interacts with a MMP-PA target molecule can be

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accomplished by any one of the methods described above
for determining direct binding. For example, determining
the ability of an MMP-PA to bind to or otherwise interact
with a MMP-PA target molecule can be accomplished by
5   determining the activity of the target molecule. For
example, the activity of the target molecule can be
determined by detecting MMP proprotein activation or
proteolysis (e.g., in the assay presented in the examples
below).

10   A cell-free assay can also be carried out by
contacting a MMP-PA, or biologically active portion
thereof, with a test compound and determining whether
(and optionally, to what extent) the test compound binds
to the MMP-PA, or the biologically active portion
15 thereof. Binding between the test compound and the MMP-
PA can be determined either directly or indirectly, as
described above. The assay can include contacting the
MMP-PA, or a biologically active portion thereof, with a
known compound that binds MMP-PA to form an assay
20 mixture, contacting the assay mixture with a test
compound, and determining the ability of the test
compound to interact with a MMP-PA protein. Thus,
determining the ability of the test compound to interact
with a MMP-PA includes determining the ability of the
25 test compound to preferentially bind to MMP-PA, or a
biologically active portion thereof, as compared to the
known compound.
        A cell-free assay can also be carried out by
contacting a MMP-PA, or a biologically active portion
30 thereof, with a test compound and determining the ability
of the test compound to modulate (e.g., stimulate or
inhibit) the activity of the MMP-PA protein, or a
biologically active portion thereof. Determining the
ability of the test compound to modulate the activity of
35 MMP-PA can be accomplished, for example, by determining

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the ability of the MMP-PA protein to bind to a MMP-PA
target molecule by one of the methods described above for
determining direct binding. Alternatively, determining
the ability of the test compound to modulate the activity
5 of MMP-PA can be accomplished by determining the ability
of the MMP-PA to further modulate a MMP-PA target
molecule. For example, one can determine the catalytic
or enzymatic activity of the target molecule on an
appropriate substrate, as previously described.
10   A cell-free assay can also be carried out by
contacting the MMP-PA, or a biologically active portion
thereof, with a known compound that binds MMP-PA to form
an assay mixture, contacting the assay mixture with a
test compound, and determining the ability of the test
15 compound to interact with a MMP-PA protein. Determining
the ability of the test compound to interact with a MMP-
PA protein can include determining the ability of the
MMP-PA protein to preferentially bind to or modulate the
activity of a MMP-PA target molecule, such as MMP-3 or
20 MMP-9.
          In any of the assays or methods described herein,
it may be desirable to immobilize either the MMP-PA or
its target molecule to facilitate separation of complexed
from uncomplexed forms of one or both of the proteins, as
25 well as to accommodate automation of the assay. Binding
of a test compound to MMP-PA, or interaction of MMP-PA
with a target molecule in the presence and absence of a
candidate compound, can be accomplished in any vessel
suitable for containing the reactants. Examples of such
30 vessels include microtitre plates, test tubes, and micro-
centrifuge tubes. The solid support used for
immobilization can include an array, such as a microtitre
plate, or beads which can be separated from a mixture.
Following incubation, the microtitre plate wells are
35 washed to remove any unbound components, and the complex

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determined either directly or indirectly, for example, as
described above. Alternatively, the complexes can be
dissociated from the solid support, and the level of MMP-
PA binding or activity determined using standard
5 techniques.

        Other techniques for immobilizing proteins on
matrices can also be used in the present screening
assays. For example, either MMP-PA or its target
molecule can be immobilized utilizing conjugation of
10 biotin and streptavidin. Biotinylated MMP-PA or target
molecules can be prepared from biotin-NHS (N-hydroxy-
succinimide) using techniques well known in the art
(e.g., biotinylation kit, Pierce Chemicals, Rockford,
IL), and immobilized in the wells of streptavidin-coated
15 96 well plates (Pierce Chemicals, Rockford, IL).
Alternatively, antibodies reactive with MMP-PA or target
molecules but which do not interfere with binding of the
MMP-PA protein to its target molecule can be derivatized
to the wells of the plate, and unbound target or MMP-PA
20 trapped in the wells by antibody conjugation. Methods
for detecting such complexes include immunodetection of
complexes using antibodies reactive with the MMP-PA or
target molecule, as well as enzyme-linked assays, which
rely on detecting an enzymatic activity associated with
25 the MMP-PA or target molecule.

        The invention encompasses the novel agents
identified by the screening assays described above, and
uses for those agents (e.g., in diagnostic and treatment
regimes), as described herein.

30   More specifically, one can use diagnostic assays
to determine MMP-PA activity in a biological sample
(e.g., a sample of blood, serum, cells, or tissue) and,
thereby, determine whether an individual has a disease or
disorder associated with aberrant MMP-PA expression or
35 activity.

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Similarly, one can use assays to determine whether an individual is at risk of developing a disease or disorder associated with aberrant MMP-PA activity. These assays can be used to identify an individual

5   prophylactically, *i.e.*, prior to the symptomatic or overt onset of a disease or disorder characterized by or associated with aberrant MMP-PA activity.

The compositions and methods described herein can be used to monitor the influence of agents (*i.e.*,

10 chemical agents or other compounds) on the activity of MMP-PA. For example, in a clinical trial of a pharmaceutical agent designed to treat or prevent an arthritic condition (*e.g.*, osteoarthritis) by inhibiting the activity of MMP-PA, one could determine the activity

15 of MMP-PA in biological samples (*e.g.*, samples of synovial fluid) that were obtained from individuals participating in the clinical trial before and at least once during the trial. A reduction in MMP-PA activity in samples obtained after treatment was begun indicates that

20 the agent administered provides an effective treatment for the arthritic condition. Similarly, a reduction in MMP-PA activity in samples obtained from patients being treated with the agent, relative to comparable samples obtained from untreated patients, indicates that the

25 agent administered provides an effective treatment for the arthritic condition.

One can detect the presence or absence of MMP-PA (and, optionally, determine the level of MMP-PA expression or activity) in a biological sample by

30 contacting the sample (*e.g.*, a sample obtained from a test subject) with a compound or an agent capable of binding to or otherwise interacting with MMP-PA in a way that reflects MMP-PA expression or activity.

One of the agents that can be used to detect MMP-

35 PA is an antibody that specifically binds to MMP-PA. For

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ease of detection, the antibody can be linked to a
detectable marker. Antibodies can be polyclonal, or more
preferably, monoclonal. The term "detectable marker," as
used in the context of an antibody probe (or other type
5 of probe for MMP-PA expression or activity), encompasses
antibodies that are directly coupled (i.e., physically
linked) to a detectable substance, as well as antibodies
that are indirectly linked to a detectable substance.
For example, an indirect indicator of MMP-PA activity is
10 a primary antibody that is detected by its ability to
bind a fluorescently labeled secondary antibody.

In vitro techniques for detecting MMP-PA include
enzyme linked immunosorbent assays (ELISAs), Western blot
analyses, immunoprecipitations, and immunofluorescence.
15 In vivo techniques for detecting MMP-PA include
introducing a labeled anti-MMP-PA antibody into a
subject. The antibody can be labeled, for example, with
a radioactive marker whose presence and location in a
subject can be detected by standard imaging techniques.

20   Those of ordinary skill in the art are accustomed
to including "control" samples in experiments and would
recognize that a biological sample from a control subject
could be manipulated in parallel with biological samples
from test subjects by, for example, contacting the
25 control and test samples (i.e., a biological sample
obtained from a subject of interest (e.g., a person who
is thought to have a disease or disorder associated with
aberrent MMP-PA expression or activity)) with a compound
or agent capable of detecting MMP-PA. MMP-PA expression
30 or activity in the control sample is then compared with
the expression or activity of MMP-PA protein in the test
sample.

Kits can be assembled for detecting MMP-PA
expression or activity in a biological sample (a test
35 and/or a control sample). These kits can be used, for

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example, to determine if a subject is suffering from or
is at increased risk of developing a disorder associated
with aberrant MMP-PA expression or activity. A kit can
include a labeled compound or agent that detects MMP-PA
5   in a biological sample (e.g., an anti-MMP-PA antibody)
and one or more reagents for determining the amount of
MMP-PA in the sample. Kits may also include instructions
(e.g., text or diagrams) useful in concluding that the
tested subject is suffering from or is at risk of
10 developing a disease or disorder associated with aberrant
expression or activity of MMP-PA. For example, the
instructions may disclose the normal range of a MMP-PA.

In the event a kit is an antibody-based kit, it
may include: (1) a first antibody (e.g., an antibody
15 attached to a solid support) that specifically binds to
MMP-PA; and, optionally, (2) a second antibody, which is
different from the first antibody, and which specifically
binds to MMP-PA or the first antibody and is conjugated
to a detectable agent. Accordingly, the kit can also
20 include components useful for detecting the detectable
agent (e.g., an enzyme or a substrate).

The kit can also include, for example, a buffering
agent, a preservative, a protein stabilizing agent, a
control sample, or a series of control samples. The
25 control sample(s) can be assayed and compared to the test
sample. The significance of such comparisons will be
evident to those of ordinary skill in the art.
Typically, each component of the kit will be enclosed
within an individual container and all of the various
30 containers will be contained within a single package
along with instructions for determining whether the
tested subject is suffering from or is at risk of
developing a disease or disorder associated with aberrant
expression or activity of MMP-PA.

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Methods of Treatment

In one aspect, the invention provides a method for preventing in a subject, a disease or condition associated with an aberrant MMP-PA expression or
5 activity, by administering to the subject an agent which modulates MMP-PA expression or at least one MMP-PA activity. Subjects at risk for a disease which is caused or contributed to by aberrant MMP-PA expression or activity can be identified by, a diagnostic assay as
10 described herein. Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of the MMP-PA aberrancy, such that a disease or disorder is prevented or, alternatively, delayed in its progression. Depending on the type of
15 MMP-PA aberrancy, for example, a MMP-PA agonist or MMP-PA antagonist agent can be used for treating the subject. The appropriate agent can be determined based on screening assays described herein.

Another aspect of the invention pertains to
20 methods of modulating MMP-PA expression or activity for therapeutic purposes. The modulatory method of the invention involves contacting a cell with an agent that modulates one or more of the activities of MMP-PA. In one embodiment, the agent stimulates one or more of the
25 biological activities of MMP-PA protein. Examples of such stimulatory agents include active MMP-PA protein. In another embodiment, the agent inhibits one or more of the biological activities of MMP-PA protein. Examples of such inhibitory agents include anti-MMP-PA antibodies.
30 These modulatory methods can be performed *in vitro* (*e.g.*, by culturing the cell with the agent) or, alternatively, *in vivo* (*e.g.*, by administering the agent to a subject). As such, the present invention provides methods of treating an individual afflicted with a disease or
35 disorder characterized by aberrant expression or activity

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of a MMP-PA protein or nucleic acid molecule. In one
embodiment, the method involves administering an agent
(e.g., an agent identified by a screening assay described
herein), or combination of agents that modulates (e.g.,
5 upregulates or downregulates) MMP-PA expression or
activity. In another embodiment, the method involves
administering a MMP-PA protein as therapy to compensate
for reduced or aberrant MMP-PA expression or activity.

        Stimulation of MMP-PA activity is desirable in
10 situations in which MMP-PA is abnormally downregulated
and/or in which increased MMP-PA activity is likely to
have a beneficial effect. Conversely, inhibition of MMP-
PA activity is desirable in situations in which MMP-PA is
abnormally upregulated and/or in which decreased MMP-PA
15 activity is likely to have a beneficial effect.

        The invention is further illustrated by the
following examples, which are provided to illustrate, not
limit, the invention. The references, patents, and
published patent applications cited throughout this
20 application are hereby incorporated by reference in their
entirety.

                         Examples

Methods

        Materials. Bovine proMMP-3 was purified from the
25 culture medium of IL-1-stimulated cartilage to a specific
activity of 550 u/mg essentially as described in
Treadwell et al. (Arch. Biochem. Biophys. 251:715-723,
1986). Human proMMP-3 purified from synovial fibroblast
conditioned medium was purchased from Accurate Chemical
30 and Scientific Corp. Link protein was purified from
bovine articular cartilage by associative and
dissociative density gradient centrifugation (Tang
et al., J. Biol. Chem. 254:10523-10531, 1979). Human
recombinant IL-1 was a gift from Peter Lomedico, of

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Hoffman LaRoche (Nutley, NJ), and human recombinant TIMP-1 was a gift from David Carmichael, of Synergen Corp. However, both these polypeptides are commercially available, for example, from CalBiochem (San Diego, CA).

5   *Preparation of cartilage media.* Articular cartilage was resected from the radial carpal joints of 7 to 11 day old calves. Cartilage from 4 joints (~40 gm) was washed with 50 volumes of sterile phosphate buffered saline (PBS) and placed in approximately 25 volumes of

10   Dulbecco's modified Eagle's medium (DMEM) containing 15 mM HEPES (pH 7.4), penicillin (100 u/ml), streptomycin (50 μg/ml), and ascorbate (50 μg/ml). Cartilage was cultured in humidified air with 5% CO_2 at 37°C. After 24 hours the medium was discarded and replaced with fresh

15   DMEM. Cultures were incubated at 37°C, and the media were harvested at 4 day intervals. Harvested media were adjusted to 0.02% NaN_3 and concentrated to dryness by the combined action of an Amicon Diaflow hollow fiber concentrator (3,000 molecular weight cutoff cartridge)

20   followed by an Amicon concentrator fitted with a YM-10 membrane. Concentrated material was solubilized in 10 ml B-50 (20 mM tris HCl pH 7.8, 1 mM $CaCl_2$, 0.1 μM $ZnCl_2$, 0.02% NaN_3, 0.05 M NaCl) per liter cartilage conditioned medium and stored at -20°C until used for purification.

25   *Protein analysis.* Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) of protein samples was carried out according to the method of Laemmli (*Nature* 22:680-684, 1970). Gelatin degrading activity was analyzed on substrate gels prepared and run

30   essentially as described by Heussen et al. (*Anal. Biochem.* 102:196-202, 1980; herein incorporated by reference in its entirety). Buffers used for purification contained 20 mM tris HCl (pH 7.8), 1 mM $CaCl_2$, 0.1 uM $ZnCl_2$, 0.02% NaN_3 with NaCl as indicated,

35   for example, B125 has 125 mM NaCl in addition to the

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components listed above. Protein concentrations were
estimated from the absorbance at 280 nm, assuming 1 A_{280}
unit is 1 mg/ml.

    Assay procedures. At each purification step,
5 fractionated material was tested for the ability to
activate bovine proMMP-3 as follows. ProMMP-3 (100 ng)
was incubated for 18 hours at 37°C with fractions to be
tested in 10 μl B-50. Activation was then assessed using
[^{14}C]-acetylated casein as a substrate for active MMP-3,
10 with the amount of casein digested in 4 hours, using 15
μl, measured essentially as previously described in
Treadwell *et al.* (*Arch. Biochem. Biophys.* 251:715-723,
1986). Appropriate controls were run in parallel to
measure activity independent of added proMMP-3. Enzyme
15 quantities at each stage of purification were adjusted to
fall within the linear range of the assay. One unit of
MMP-PA is defined as the amount of protein required to
activate 50 ng of proMMP-3 in this assay. ProMMP-3 was
activated by incubation with 1 mM APMA for 18 hours at
20 37°C to define full activation.

    For the analysis of proteolytic processing of the
MMP-3 zymogen, proMMP-3 was incubated with enzyme
fraction to be tested for 18 hours at 37°C, and the
extent of conversion from latent to lower molecular
25 weight forms was estimated following resolution of
reaction products by SDS-PAGE. Where indicated, a 4-fold
molar excess of human recombinant TIMP-1 relative to MMP
was included in the activating incubation mixture to
inhibit MMP-dependent autocatalytic cleavage.

30   *Purification of MMP-PA*. Pooled concentrated
cartilage conditioned medium (250 ml, approximately 1000
mg) was adjusted to 40% saturated NH_4SO_4, the pH was
adjusted to 7.0, and the protein was precipitated on ice
for 2 hours. Precipitated protein was removed by
35 centrifugation (12,000 x g, for 30 min at 4°C), and the

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supernatant was adjusted to 90% saturation with NH_4SO_4. Protein was allowed to precipitate overnight at 4°C, and the precipitates were recovered by centrifugation at 12,000 x g for 30 minutes at 4°C. Protein was dissolved
5 in 10 ml B-125 and dialyzed (3,500 molecular weight cutoff) against the same buffer prior to application to DEAE Sephadex A50 resin equilibrated in B125 (50 ml resin). The column flow through (5 column volumes B-125) was concentrated by Amicon to apparent dryness. The
10 concentrate was dissolved in 5 ml B-50 and applied to a 5 ml Matrex Gel Green A (Amicon Corp.) column equilibrated in B-50.

The Matrex Gel Green flow through was collected, concentrated to apparent dryness and dissolved in 1 ml B-
15 50, and applied to a Sephacryl S-200 HR column (1.5 x 85 cm), equilibrated and run in B-50 at a flow rate of 1 ml/min. Fractions (2 ml) were monitored for MMP-PA by incubating 100 ng of proMMP-3 with 0.5 μl of each column fraction for 18 hours at 37°C and then measuring the
20 amount of casein digested in 4 hours. Fractions from the Sephacryl S-200 HR column showing evidence of proMMP-3 activation in the initial screen were assayed at various dilutions as described above to determine units of activity. The fraction with the highest proMMP-3-
25 activating activity was used for further characterization of MMP-PA.

Substrate specificity of MMP-PA. Various purified proteins (bovine serum albumin, link protein, plasminogen, and casein; 0.3 to 0.6 μg) were incubated in
30 15 μl B-50 with 22 ng MMP-PA for 18 hours at 37°C. For comparison, parallel incubations were carried out with protein alone or protein incubated with trypsin (22 ng). Reaction products were reduced and analyzed by SDS-PAGE.

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_NH₂-terminal sequence analysis of bovine proMMP-3
and link cleavage products generated during incubation
with MMP-PA_. Bovine proMMP-3 (8 μg) was incubated for 18
hours at 37°C with 275 ng of MMP-PA and 12.7 μg human
5   rTIMP-1 in 150 μl B-50. Bovine link protein (8 μg) was
incubated for 18 hours at 37°C with 165 ng MMP-PA in a 50
μl reaction. Digestion products were resolved by SDS-PAGE
under reducing conditions and transferred to
poly(vinylidene difluoride) membrane (PVDF) (Matsudaira,
10 _J. Biol. Chem._ 262:10035-10038, 1987). After staining
the transferred proteins with Coomassie Blue R-250, bands
corresponding to the major proteolytic fragments were
excised from the blot and sequenced using an Applied
Biosystems Model 477A Protein Sequencer.

15   _Sensitivity to thermal denaturation and
inhibitors_.   MMP-PA (28 ng) was incubated at various
temperatures for 20 minutes prior to testing its capacity
to activate 300 ng bovine proMMP-3 in 18 hours at 37°C.
The effect of proteinase inhibitors on proMMP-3
20 activation was similarly tested except that there was no
preincubation step. Subsequent to the activating
incubations, active MMP-3 was measured using [¹⁴C]casein
as a substrate in a 4 hour reaction.

        The effect of chelating agents on MMP-PA
25 proteolysis of bovine proMMP-3 and link protein was
assessed by comparison of reaction products following
SDS-PAGE. Recombinant TIMP-1 was included during the
incubation to limit MMP-dependent proteolysis. To
further explore the metal dependence of MMP-PA
30 proteolytic activity, the enzyme buffer was first
exchanged with 20 mM Tris HCl pH 7.8, 50 mM NaCl, 0.02%
NaN₃ (TBS) by repeated dilution and concentration using a
Nanosep centrifugal concentrator (10,000 molecular weight
cutoff, Pall Corp.).

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Table 1 summarizes the procedure used to isolate
proMMP-3-activating activity from bovine articular
cartilage conditioned medium. Twenty-five liters of
explant culture medium (1000 A280 units) yielded 22 μg
5 protein at the S-200 stage of purification with a 2500-
fold increase in specific activity over the starting
conditioned medium.

Table 1. Purification of MMP-PA					
Step	Total protein	Total Activity	Yield	Specific Activity	Purification
	mg.	units*	%	units/mg	-fold
Crude**	1,000	187,500	-	187	-
DEAE	10.1	163,000	87	16,300	87
Green Matrex	0.4	8,700	4.6	21,750	116
S-200	0.022	10,200	5.4	463,600	2,479

At each stage of purification, active material was
analyzed by SDS-PAGE and zymography on casein and gelatin
10 substrate gels in order to trace the removal of contaminants.
The NH$_4$SO$_4$ fractionation removed the bulk of the
proteoglycans, which otherwise interfered with subsequent
purification steps, and some of the carboxy-terminal
propeptide of type II collagen (the major protein in
15 cartilage conditioned medium). Chromatography on DEAE-
Sephadex removed most of the remaining proteoglycans and a 57
kDa casein degrading activity (presumably proMMP-3). The
Matrex Gel Green resin removed most of the gelatinase
activity (MMP-2 and MMP-9), the remaining C-propeptide, and
20 many low abundance proteins.

In Table 1, one "unit" is the amount of protein required
to activate 50 ng proMMP-3, as determined by digestion of
[^{14}C]casein using the assay described above (see "Methods"),
and the row labeled "crude" refers to total protein in
25 concentrated medium. Activity at early stage cannot be accurately measured because of interference from other

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components (other proteinases, substrates, and
inhibitors).

　　　The proMMP-3 activating activity eluted from
Sephacryl S-200 with an apparent molecular weight of
5　about 43,000 (Fig. 1A). The proteins in each fraction
were resolved on SDS polyacrylamide gels under reducing
conditions, and the protein bands were visualized by
silver staining (Fig. 1B). Aliquots of Sephacryl S-200
column fractions spanning the peak of activity (fractions
10　34-41) were incubated with proMMP-3, and activation
products were resolved on a SDS polyacrylamide gel (Fig.
1C). Visual inspection of the gel profiles revealed that
the major peak of proMMP-3-activating activity (fractions
35-38) paralleled the intensity of bands corresponding to
15　a "doublet" at about 72 kDa and a somewhat diffuse band
at about 52 kDa. Active fractions from the S-200 column
(5 μl) were tested for endogenous "caseinase" and
"gelatinase" activity in the absence of added proMMP-3.
Sephacryl S200 fraction number 37, lacking detectable
20　gelatinase or caseinase activity, was used to further
characterize MMP-PA.

　　　Time and dose-dependent activation of proMMP-3 by
MMP-PA. Human proMMP-3 was predominantly in the latent
form, migrating as a 59/57 kDa doublet on SDS-PAGE. Some
25　activation of latent enzyme occurred during incubation in
the absence of exogenous activator, as indicated by the
increased digestion of the [^{14}C]-labeled casein substrate
with time of incubation (Fig. 2A). MMP-PA itself had no
caseinase activity detectable by this assay. After
30　incubation of the MMP-3 zymogen with MMP-PA, a marked
increase in the cleavage of labeled casein was evident.
Activation of proMMP-3 was evident after 1 hour
incubation with 1 μg/ml MMP-PA (Fig. 2).

　　　Stepwise processing of proMMP-3 by MMP-PA. In
35　order to allow the accumulation of activation

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intermediates generated by MMP-PA, activation reactions
were carried out in the presence of TIMP-1. At least
three species in the size range 45,000 to 53,000 daltons
were detected when reaction products were resolved by
5 SDS-PAGE (Fig. 3, lanes 4-6).

Substrate specificity of MMP-PA proteolysis. In
order to further investigate the specificity of MMP-PA,
various proteins were incubated with the enzyme prior to
analysis of digestion products by SDS-PAGE. After
10 incubation under conditions that completely converted
proMMP-3 to an active form, the electrophoretic
mobilities of bovine serum albumin and plasminogen were
unchanged. However, cartilage link protein was cleaved
to generate a major reaction product slightly smaller
15 than the pristine molecule. By comparison, trypsin
degraded all of these substrates to sub-18 kDa
polypeptides that were electrophoresed from the gel.

Definition of MMP-PA cleavage sites in proMMP-3
and link protein. To define more clearly the substrate
20 specificity of MMP-PA's proteolytic activity, the
sequences of the amino termini of the major digestion
products of proMMP-3 and link protein were determined
(Table 2).

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Table 2. NH$_2$-terminal sequences of digestion products of bovine proMMP-3 and link protein accumulating during incubation with MMP-PA.							
	Bovine proMMP-3					Bovine Link Protein	
Cycle	Sequence 1*					Sequence 2	
1	R		K			R	
2	K		P			V	
3	P		R			I	
4	R		X			H	
5	X		G			I	
6	G		I			Q	
7	I		P			A	
8	P		X			E	

        * The proMMP-3 digestion product gave a mixed
sequence. The two sequences were resolved by comparison
with the sequence for human proMMP-3.

        This analysis demonstrated that bovine proMMP-3
was cleaved during incubation with MMP-PA at sites
corresponding in the human homolog to the methionine$_{70}$-
arginine$_{71}$ and arginine$_{71}$-lysine$_{72}$ bonds in the propeptide
to create two 46,000 M_r products that appeared as a single
band on the Coomassie Blue stained blot (Fig. 4). The N-
terminal sequence of the unique link digestion product
detected indicated that MMP-PA cleaved the aspartic
acid$_{12}$-arginine$_{13}$ bond to generate about a 42 kDa
catabolite.

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Functional characterization. MMP-PA was stable to
incubation at 55°C, partially inactivated at 60°C, and
completely inactivated at 70°C.

Bovine proMMP-3 (300 ng) was incubated with 28 ng
5 MMP-PA for 18 hours with each of the additives shown in
Table 3. Active MMP-3 was measured using [^{14}C]-casein as
a substrate. ProMMP-3 activation by MMP-PA was inhibited
by 4-(2-aminoethyl)-benzenesulfonyl-fluoride (AEBSF) and
benzamidine, but the enzyme was not effectively inhibited
10 by any other serine proteinase inhibitor tested (*see*
Table 3). The enzyme was not inhibited by agents that
target members of the aspartic or cysteine class of
proteinases (E-64, N-ethyl maleimide, pepstatin).

Table 3. Sensitivity of MMP-PA to inhibitors.		
INHIBITOR	**CONCENTRATION**	**INHIBITION (%)***
Benzamidine	0.8 mM	67
Benzamidine	1.6 mM	84
AEBSF	1 mM	100
PMSF	1 mM	25
DCIC	0.2 mM	0
TLCK	0.14 mM	0
aprotinin	0.3 μM	11
SBTI	5 μM	●
α1 PI	0.2 μM	4
leupeptin	1 μM	2
E-64	25 μM	6
pepstatin	1 μM	13
NEM	1 mM	0

Peptide bond specificity of MMP-PA. Peptide bonds
30 hydrolyzed by MMP-PA in proMMP-3 and link protein (arrow)

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are aligned in Table 4 to show similar or identical amino acids at five positions in the decapeptides that encompass the cleavage sites in the two substrates. Shown below for comparison are sites with arginine at P_1'
5 in various peptides cleaved by N-arginine dibasic convertase (NRD) or insulin degrading enzyme (IDE) (compiled from Chesneau et al., J. Biol. Chem. 269:2056-2061, 1994, and Muller et al., Biochem. 31:11138-11143, 1992).

10 Table 4.

MMP-PA

        DSDTLEVM RK   proMMP-3
        | ||| |
        DNYTLDHD RA   link
15   ⇑
    NRD

        NPAMAPRE RK   somatostatin-28
        PRRPYILK RA   preproneurotensin
            YGGFLR RI   dynorphin A
20   QNCPLGGK RA   pro-ocytocin/neurophysin

    IDE

        GLGCNVLR RY   brain natriuretic peptide
            DSGCFG   RR   brain natriuretic peptide
            FGRRLD   RI   brain natriuretic peptide
25   CFGLKLD RI   C-type natriuretic peptide
            FGGRID   RI   atrial natriuretic peptide

    Propeptide catabolites can serve as novel indicators of progressive cartilage loss. As described above, the progressive destruction of cartilage at the
30 articular surfaces results from increased proteolytic

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activity. The novel proteinase described herein, which
activates MMP-3 (stromelysin) and MMP-9 (92-k
gelatinase), is produced by articular cartilage and may
regulate MMP activity in the avascular tissues of the
5 joint not only during development, but also in the
processes of growth, repair, and disease. Excessive
activity of MMP-PA in conditions such as osteoarthritis
would disrupt the tight regulation of ECM degrading
enzymes and, ultimately, cause cartilage destruction.
10 The compositions and methods described herein can be used
to "fingerprint" MMP-PA activity in biological samples,
and thereby detect and diagnose conditions in which
tissue is destroyed.

In addition to activating MMP-3 and MMP-9, MMP-PA
15 may activate an entire degradative cascade in cartilage.
To examine the extent of MMP-PA involvement, MMP-3, -9, -
13, and "aggrecanase" proenzyme catabolites will be
examined in cartilage and synovial fluids for terminal
amino acid sequence consistent with *in vivo* activity of
20 MMP-PA. These MMPs are present in adult cartilage and
zymogen activation is the rate-limiting step in
proteolysis catalyzed by these enzymes (*i.e.*, MMP-3, -9,
-13, and "aggrecanase" proenzymes).

Catabolites generated during activation of these
25 proenzymes are strongly correlated with progressive
degenerative pathologies. Thus, by identifying specific
catabolites in the synovial fluid of affected patients,
one can monitor and define critical steps in disease
progression and assess the effect of therapeutic agents
30 aimed at slowing, halting, or preventing that progress.

Several MMPs, including MMP-1, MMP-3, MMP-9, and
MMP-13, are up-regulated by IL-1 in cartilage. Moreover,
once the zymogen forms are activated, they are capable of
digesting the major matrix components (Treadwell *et al.*,
35 *Arch. Biochem. Biophys.* 251:715-723,1986; Stephenson *et*

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al., *Biochem. Biophys. Res. Commun.* 144:583-90, 1987; Mohtai *et al.*, *J. Clin. Invest.* 92:179-185, 1993; Borden *et al.*, *J. Biol. Chem.* 271:23577-81, 1996; Reboul *et al.*, *J. Clin. Invest.* 97:2011-2019, 1996). Activation of MMP

5   zymogens involves the removal of a conserved N-terminal propeptide, most likely through proteolytic cleavage. Since these enzymes are secreted and freely diffusible through the extracellular matrix, zymogen activation at the cell surface could localize their activity. MMP-3

10   and MMP-9 may be especially important for cartilage degradation. Active forms of these enzymes are generated in explant cultures of osteoarthritic cartilage (Mohtai *et al.*, *J. Clin. Invest.* 92:179-185, 1993; Dean *et al.*, *J. Clin. Invest.* 84:678-685, 1989). Such explant

15   cultures are among those that can be used to assay modulators of MMP-PA activity (*e.g.*, molecules or compounds that alter the expression or activity of MMP-PAs). In addition, degradation products consistent with cleavage at sites targeted by MMPs are present in

20   cartilage and synovial fluid obtained from patients with osteoarthritis.

        MMPs are normally tightly regulated by the requirement for zymogen activation. Despite the low turnover of normal adult articular cartilage, MMPs are

25   expressed in both normal and osteoarthritic cartilage. Adult cartilage explants, whether normal or osteoarthritic, undergo rapid degradation in response to the MMP activator p-aminophenyl mercuric acetate. The major aggrecan catabolites result from cleavage at the

30   MMP site (Flannery *et al.*, *J. Biol. Chem.* 267:1008-1014, 1992). Thus, a substantial pool of proMMPs exists in adult cartilage, which further suggests that zymogen activation is the rate-limiting step in degradation catalyzed by these enzymes.

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Depletion of chondroitin sulfate-rich aggrecan catabolites, which is the earliest event in matrix degradation, is catalyzed not by any of the well-characterized metalloproteinases but by "aggrecanase,"

5 which cleaves aggrecan between glutamic acid and alanine in the interglobular domain and, perhaps, elsewhere (Sandy *et al.*, *J. Biol. Chem.* 266:8683-8885, 1991; Lark *et al.*, *Biochem. J.* 307:245-252, 1995; Loulakis *et al.*, *Biochem. J.* 284:589-593, 1992; Ilic *et al.*, *Arch.*

10 *Biochem. Biophys.* 294:115-122, 1992). Aggrecan degrading metalloproteinase (aggrecanase) is synthesized as a precursor but may be secreted in active form. Analysis of the ADMP sequence revealed a cysteine switch-like sequence and a potential furin-cleavage site (polybasic

15 region), suggesting a typical though perhaps intracellular mechanism of proenzyme activation.

MMPs may be involved later in the progression of cartilage degradation. For example, they may catalyze secondary cleavages of aggrecan and other non-collagenous

20 components of cartilage and, perhaps more importantly, catalyze the destruction of collagen. Aggrecan molecules that have been lost can be newly synthesized and thereby replaced, but the destruction of the collagen framework is irreversible.

25   The foregoing demonstrates that distinct phases occur during the loss of articular cartilage. Aggrecanase acts earlier to release the chondroitin sulfate-rich aggrecan fragments from their hyaluronan anchor in the matrix, and the MMPs (collagenases,

30 gelatinases, and stromelysins) are activated later to trigger the "point of no return," where excessive catabolism of structural elements overwhelms the chondrocyte's attempts to repair the damaged extracellular matrix. The rate limiting step in the

35 damage catalyzed by these enzymes is zymogen activation.

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The MMP-PA described herein directly activates the MMP-3 and MMP-9 proenzymes by cleaving at specific sites in the propeptides. Because MMP-3 can activate or contribute to the activation of several other MMPs,

5   including MMP-1, MMP-8, and MMP-13 (collagenases) and MMP-9 (92 k gelatinase), MMP-PA has the potential to initiate a cascade of extracellular matrix degrading MMPs (such cascades are described, e.g., by Knauper et al., Biochem. J. 295:581-586, 1993; Knauper et al., J. Biol.

10 Chem. 271:1544-1550, 1996; Nagase et al., in Joint Cartilage Degradation: Basic and Clinical Aspects, J.F. Woessner and D.S. Howell (Eds.), Marcel Dekker, Inc., 1993). Increased MMP-PA activity would lead to excessive catabolism of extracellular matrix under pathologic

15 conditions, such as osteoarthritis, and plays a role at multiple steps in the progressive degeneration of osteoarthritis.

A stepwise mechanism for the activation of MMP zymogens has been described in which a proteinase

20 initially cleaves within a proteinase-susceptible region of the propeptide (Nagase et al., in Joint Cartilage Degradation: Basic and Clinical Aspects, J.F. Woessner and D.S. Howell (Eds.), Marcel Dekker, Inc., 1993). This initial activation product is then converted in

25 bimolecular "autocatalytic" reaction(s) to a more stable active form having a phenylalanine or tyrosine NH_2-terminus. Similar stepwise processing through a transient 53 kDa activation intermediate occurred upon incubation of proMMP-3 with MMP-PA. In the presence of

30 TIMP, which inhibits the autocatalytic conversion of the 53 kDa species to the 45 kDa species, a 46 kDa product accumulated to levels sufficient for N-terminal sequence analysis. A dibasic site in the proMMP-3 molecule was targeted under these conditions, with cleavage occuring

35 on the amino side of an arginine residue. This cleavage

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is secondary to a cleavage that creates a transient
activation intermediate (~53 kDa), a size consistent with
cleavage at another basic region closer to the amino
terminus. A polybasic sequence reportedly exists in the
5 aggrecanase propeptide (postulated furin cleavage site),
and MMP-PA may target that site. Proteinase-laden
membranous packets (matrix vesicles, apoptotic bodies,
podosomes) are released from chondrocytes in the later
degenerative phase of osteoarthritis. The precise origin
10 of these extravasated membranes is unknown. It is
possible that MMP-PA is localized intracellularly within
endosomes, where it catalyzes proAggrecanase activation
early in the process of articular cartilage loss, and
that release of these structures as apoptotic bodies
15 later in the degenerative process results in activation
of secreted proMMPs (Hashimoto *et al.*, *Arth. and Rheum.*
41:1632-1638, 1998; Yang *et al.*, *Exp. Cell Res.* 235:370-
373, 1997).

    The examples below describe: (1) creation of
20 tagged synthetic propeptide-like substrates that can be
used to assay and characterize proenzyme activator(s)
endogenous to osteoarthritic cartilage (including MMP-
PA); (2) use these polypeptides in osteoarthritic
cartilage explants and purified systems to define sites
25 of natural and MMP-PA-catalyzed activating cleavages in
the MMP-3, MMP-9, and MMP-13 propeptides (and perhaps
proAggrecanase); and (3) production of neoepitope,
capture, and detection reagents useful for ELISA-based
measurement of proenzyme catabolites in synovial fluid.
30 These reagents can be used not only to clarify the role
of MMP-PAs in regulating enzymes that become activated in
osteoarthritis and other degenerative conditions, but
also to evaluate proenzyme catabolites as markers for
critical steps in the progression of these conditions.

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1. *Expression of recombinant MMP-3, MMP-9, MMP-13, and Aggrecanase propeptides to assay putative MMP activators and to further define pathology-related and MMP-PA-catalyzed cleavage site(s).* It is of
5   considerable value to define the initial sites that are cleaved in the stepwise activation of zymogens in an explant culture system of osteoarthritic cartilage. It is also of value to define the site targeted by MMP-PA to generate the transient 53 kDa MMP-3 activation
10 intermediate that is subsequently clipped to create a phenylalanine or tyrosine N-terminus and to identify cleavages in the MMP-9 and MMP-13 propeptides. To avoid secondary MMP-dependent "autocatalysis," bacteria are used to express recombinant propeptides that are
15 truncated at the junction to the catalytic domain. These propeptide sequences have carboxy terminal hexahistidine tails to enable purification on nickel resin or adherence to metal chelate plates. An epitope tag at the N-terminus allows immunodetection of full length plate-
20 bound propeptide but not propeptide that has been clipped. These tagged recombinant propeptides can be used to develop ELISA-based assays of clipping activity (which reflects proMMP-activating activity) in biological fluids such as cartilage extracts or explant culture
25 media. These assays can also be used to analyze the clipping activity of purified enzymes, or to assess the biological activity of fragments thereof.

In addition, ELISA-based assays are useful to define sites of proteolysis within the propeptide in the
30 absence of secondary bimolecular "autocatalysis," since catabolites with newly generated N-termini can be conveniently purified for sequencing. Comparison of propeptide cleavage sites in an osteoarthritic cartilage explant system with sites cleaved by MMP-PA will clarify
35 the role of this enzyme in MMP proenzyme activation.

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This information will be used to create neoepitope
antisera, as described below. This approach will define
sites cleaved in the natural pathology and allow the
development of appropriate reagents to detect these
5 catabolites.

        2.   *Preparation of antisera to the N-terminal
neoepitopes generated by proteolysis of the MMP-3, MMP-9,
MMP-13, and Aggrecanase propeptides.* Neoepitope
technology is based on newly created epitopes (new amino
10 and carboxy terminal ends) generated when a protein is
cleaved. Antibodies raised against synthetic peptides
corresponding to these epitopes are utilized in the
identification or detection of these cleaved products in
biological fluid. Therefore, as with protein fragment
15 sequencing, proteinase(s) responsible for specific
cleavages are identified indirectly. This method has
been used to identify matrix metalloproteinase-cleaved
polypeptides amongst aggrecan digestion products in
cartilage tumors (Toriyama *et al.*, *Eur. J. Cancer*
20 <u>34</u>:1969, 1998).

        Antisera will be prepared to detect propeptide
catabolites generated by specific clipping at MMP-PA
cleavage sites. Synthetic peptides corresponding to the
N-terminal neoepitopes (for example KDVKQFV (SEQ ID
25 NO:1), RRKDSGP (SEQ ID NO:2), SDTLEVM (SEQ ID NO:3), and
RKPRCGV (SEQ ID NO:4) in the proMMP-3 propeptide and
GYTRVAEM (SEQ ID NO:5), RGESKSL (SEQ ID NO:6), SATLKAM
(SEQ ID NO:7), and RTPRCGV (SEQ ID NO:8) in the proMMP-9
propeptide) will be crosslinked to keyhole limpet
30 hemocyanin through a C-terminal cysteine residue. The
antiserum is raised by immunizing rabbits against the
carrier-peptide conjugate. The protocol employs
immunization with 250 μg in complete Freund's adjuvant
followed by at least three boosts of the same immunogen

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(100 μg) in incomplete Freund's. Rabbit bleeds are
assessed by ELISA using peptides and by Western blotting
of activation intermediates of the proenzymes generated
in explant cultures or by MMP-PA in partial reactions.
5 Antisera will react with the appropriate digests, but not
with undigested proenzyme or enzyme activated by other
agents, such as trypsin. Immunoreactive signals can be
abrogated by preincubation of the antiserum with the
full-length immunizing peptide but not by preincubation
10 with truncated peptides. Initial characterization of
these antisera will be followed by further ELISAs using
permutations of the amino acids contained in or near the
cleavage site.

     In addition to the neoepitope reagents, a
15 universal detection antiserum will be produced to
specifically detect activation products in the microwell
plates. The target for the detection antiserum will be a
synthetic peptide based on the highly conserved cysteine
switch region at the carboxy terminus of the MMP
20 propeptides (RKPRCGVPDVC (SEQ ID NO:__). Antiserum to
the KLH-linked peptide will be prepared and tested as
above, except that reactivity exclusively with the
terminus is not required. The antiserum is likely to
recognize not only most of the MMP propeptides, but also
25 the aggrecanase propeptide. Once characterized, the
antibodies can be used to identify catabolites in
biological samples (e.g., cartilage and synovial fluids)
from osteoarthritic patients by Western blot analysis or
ELISA. Antisera production can be performed using
30 techniques routinely employed by one of ordinary skill in
the art (or performed by a commercial service), and
specific antibodies can be purified by peptide affinity
chromatography.

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3. *Development of ELISAs for the detection of metalloproteinase propeptide catabolites.* Once characterized, the reagents described above will be used to develop an ELISA for detecting and quantitating
5   relevant catabolites in biological samples obtained from patients with osteoarthritis. The capture antisera specific for neoepitopes (IgG fraction or affinity purified antibodies) will be bound to Nunc ELISA plates in carbonate buffer. Synovial fluid (or other biological
10 specimens) or standards (recombinant polypeptides) will be diluted in tris-buffered saline (TBS, 0.02 M Tris HCl, 150 mM NaCl, pH 7.5). Plates will be incubated for 1 hour at 22°C to allow capture of the propeptide catabolites. Plates will be washed with TBS, and the
15 universal detection antiserum-horse radish peroxidase conjugate will be added. After 1 hour at 22°C, plates will be washed, a chromogenic substate added, and the color detected by microplate reader.

Unlike the formidable task of purifying natural
20 catabolites from multiple specimens of osteoarthritic cartilage or synovial fluid for protein sequencing, the indirect approach described herein will allow detection and measurement of specific catabolites of proenzyme activation, which in turn serve as markers for the
25 progression of any condition in which articular cartilage is lost.

4. *Analysis of propeptide catabolites in cartilage and synovial fluid from osteoarthritic human joints as markers of pathogenesis.* Neoepitope technology
30 and ELISAs can be used to determine whether proMMPs, such as proMMP-3, proMMP-9, and proMMP-13 are activated by cleavage at sites defined above in the context of progressive pathogenesis. Analysis can be performed in synovial fluid specimens obtained from patients, for

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example, patients undergoing arthroscopic examination or joint replacement surgery. Samples will be centrifuged to remove blood cells, and the supernatants will be frozen in aliquots at -70°C. Fluids will be treated with
5 hyaluronidase in the presence of a cocktail of proteinase inhibitors. Aliquots of fluid will be diluted for assay by ELISA for specific propeptide catabolites. Especially in the case of proAggrecanase, which may be activated intracellularly, alternative biololgical fluids would be
10 considered such as extracts of cartilage (e.g., articular cartilage specimens could be excised at the time of total joint replacement). Cartilage can be snap frozen and stored at -80°C. Frozen specimens are then lyophilized and extracted in 10 volumes of hypotonic extraction
15 buffer (40 mM KCl, 20 mM Tris HCl, 0.5% Triton X-100 plus a cocktail of protease inhibitors). Extracts are then assayed by ELISA, and standard curves are prepared using the appropriate recombinant propeptides.

These studies will define proenzyme catabolites
20 (and specifically MMP-PA-generated catabolites) useful as a markers of progressive pathogenesis (e.g., progressive osteoarthritis). The values obtained for synovial fluid (and blood) can be compared against the MRI grading of the extent of disease (as well as normal controls) and
25 against the histological-histochemical grading of the extent of the disease in small segments of tissue which will, thereby, validate the assay of MMP-PA generated catabolic products in serum or synovial fluid as a screen for conditions in which articular cartilage is lost. In
30 addition, these catabolic products can be used to assess the extent of the disease.

What is claimed is:

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1. A substantially pure polypeptide, the polypeptide
being a proteinase that:

     (a) activates a matrix metalloproteinase proenzyme
(MMP);

5   (b) is produced by articular cartilage;

     (c) lacks caseinase and gelatinase activity;

     (d) does not degrade bovine serum albumin (BSA) or
plasminogen; and

     (e) is inhibited by 4-(2-aminoethyl)-
10 benzenesulfonyl-fluoride (AEBSF) or benzamidine.

2. The polypeptide of claim 1, the polypeptide being
60-70 kDa in size.

3. The polypeptide of claim 1, the polypeptide being
stable at 55°C and inactivated at 70°C.

15   4. The polypeptide of claim 1, wherein the MMP is MMP-
3.

5. The polypeptide of claim 3, wherein the polypeptide
cleaves human proMMP-3 between the methionine$_{70}$-arginine$_{71}$
and arginine$_{71}$-lysine$_{72}$ bonds.

20   6. The polypeptide of claim 1, wherein the MMP is MMP-
9.

7. The polypeptide of claim 1, wherein the polypeptide
is a human polypeptide.

8. A pharmaceutical composition comprising the
25 substantially pure polypeptide of claim 1 and a
physiologically acceptable carrier therefor.

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9. A substantially pure antibody that specifically binds to the polypeptide of claim 1.

10. The antibody of claim 9, wherein the antibody is a monoclonal antibody.

5   11. The antibody of claim 9, wherein the antibody inhibits the activity of the polypeptide of claim 1.

12. A pharmaceutical composition comprising the antibody of claim 9 and a physiologically acceptable carrier therefor.

10   13. A substantially pure antibody that specifically binds to a neoepitope of a polypeptide of claim 1.

14. A substantially pure polypeptide consisting of an amino acid sequence selected from the group consisting of SEQ ID NO:1 (KDVKQFV), SEQ ID NO:2 (RRKDSGP), SEQ ID NO:3

15 (SDTLEVM), and SEQ ID NO:4 (RKPRCGV).

15. The polypeptide of claim 14, further comprising keyhole limpet hemocyanin.

16. A substantially pure antibody that specifically binds a polypeptide of claim 14.

20   17. A substantially pure polypeptide consisting of an amino acid sequence selected from the group consisting of SEQ ID NO:5 (GYTRVAEM), SEQ ID NO:6 (RGESKSL), SEQ ID NO:7 (SATLKAM), and SEQ ID NO:8 (RTPRCGV).

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18. The polypeptide of claim 17, further comprising keyhole limpet hemocyanin.

19. A substantially pure antibody that specifically binds a polypeptide of claim 17.

5   20. A method for detecting or monitoring the progression of a condition associated with the loss of articular cartilage, the method comprising contacting a biological sample obtained from a patient with an antibody of claim 9, claim 16, or claim 19.

10   21. The method of claim 20, wherein the condition associated with the loss of articular cartilage is osteoarthritis.

22. The method of claim 20, wherein the biological sample is a sample of cartilage or synovial fluid.

15   23. The method of claim 20, wherein interaction between an antibody of claim 16, or claim 19, and a MMP catabolite in the biological sample is detected by an enzyme-linked immunosorbent assay or by Western blotting.

24. A method for detecting the presence of a
20 MMP-proenzyme activator (MMP-PA) in a biological sample, the method comprising contacting the biological sample with an anti-MMP-PA antibody.

25. A method for inhibiting the activity of an MMP-proenzyme activator (MMP-PA), the method comprising
25 contacting a biological sample with an anti-MMP-PA antibody or a small molecule.

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26. A method for treating a patient having a condition associated with the loss of articular cartilage, the method comprising administering to the patient a compound that inhibits the activity of an MMP-proenzyme activator
5 (MMP-PA).

27. The method of claim 26, wherein the condition associated with the loss of articular cartilage is osteoarthritis.

28. The method of claim 26, wherein the compound that
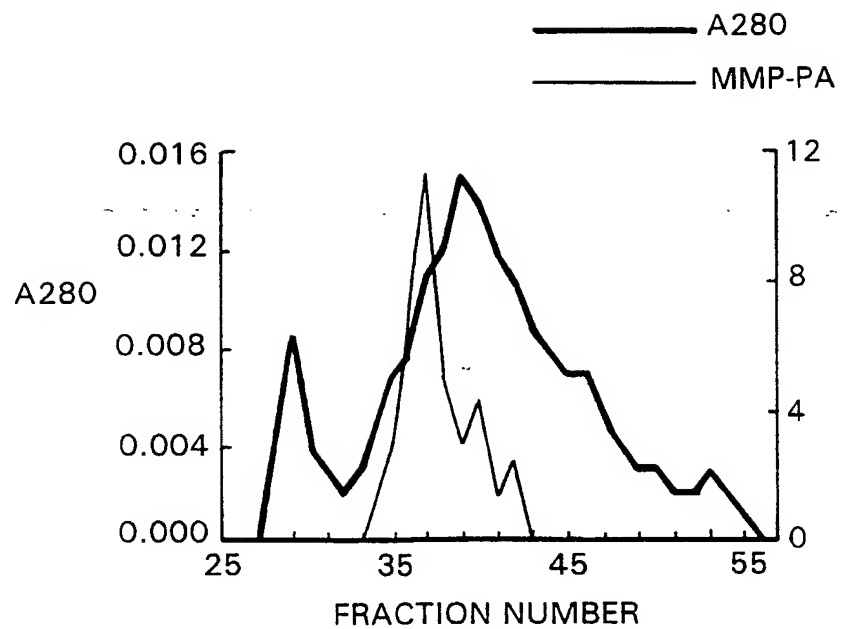10 inhibits the activity of MMP-PA is an antibody or small molecule that specifically binds MMP-PA.

29. A method for stimulating the loss of articular cartilage, the method comprising contacting the articular cartilage with an MMP-proenzyme activator (MMP-PA) or a
15 biologically active fragment thereof.

30. The method of claim 29, wherein the MMP-PA is a polypeptide of claim 1 or a biologically active fragment thereof.

31. A method for identifying a compound that modulates
20 the activity of a MMP-proenzyme activator (MMP-PA), the method comprising examining the ability of MMP-PA to activates a matrix metalloproteinase (MMP) in the presence and absence of the compound, and identifying those compounds that alter the abiltiy of MMP-PA to
25 activate MMP.

32. The method of claim 31, wherein the MMP is MMP-3 or MMP-9.

FIG. 1A

FIG. 1B

FIG. 1C
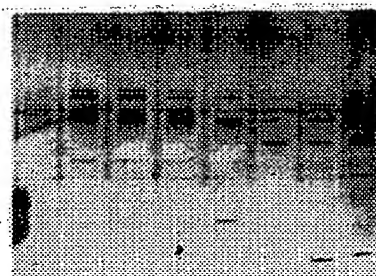
34 35 36 37 38 39 40 41

1 2 3 4 5 6
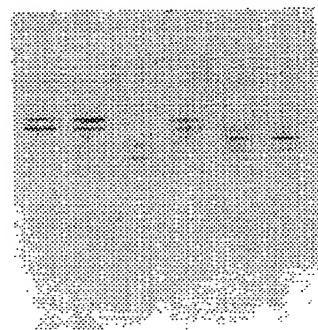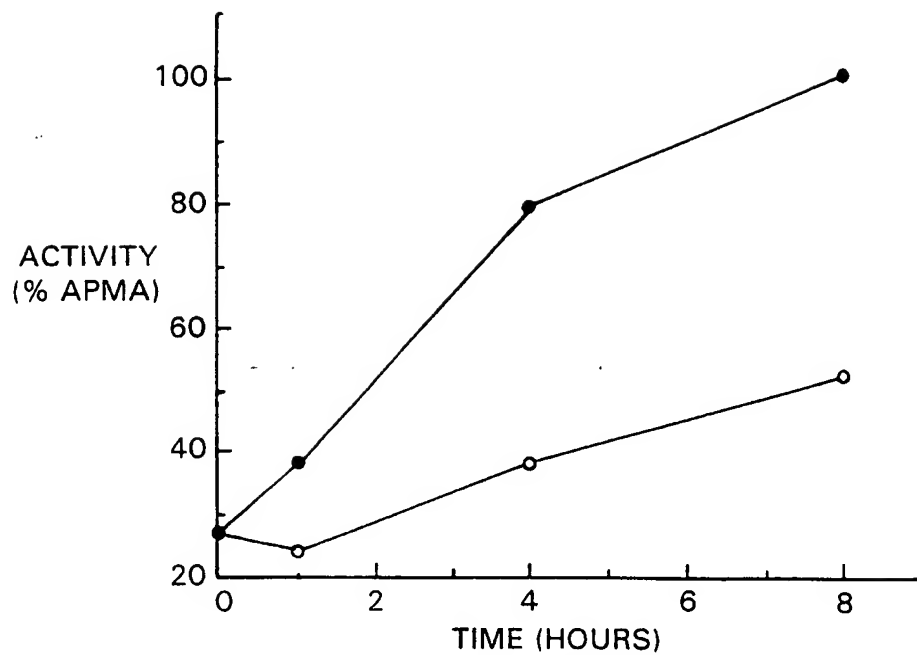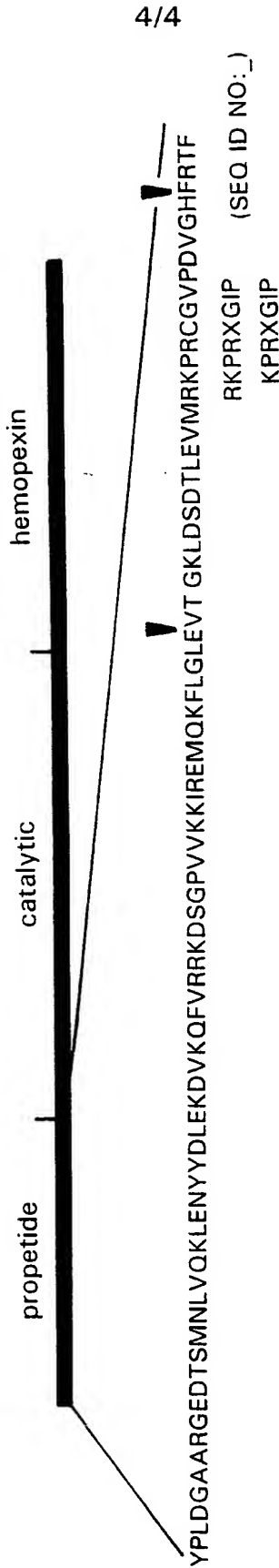
91-
69-
46-

30-

FIG. 3

FIG. 2A

FIG. 2B

FIG. 4

propetide   catalytic   hemopexin

YPLDGAARGEDTSMNLVQKLENYYDLEKDVKQFVRRKDSGPVVKKIREMQKFLGLEVT GKLDSDTLEVMRKPRCGVPDVGHFRTF

RKPRXGIP   (SEQ ID NO:_)

KPRXGIP

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 39/00, 39/395, 38/00; C07K 16/00; C12N 9/64
US CL : 424/146.1, 184.1; 435/226; 530/387.1, 323
According to International Patent Classification (IPC) or to both national classification and IPC

B.   FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. :   424/146.1, 184.1; 435/226; 530/387.1, 323

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, DIALOG, EMBASE, LIFESCI, BIOSIS, CHEM ABS, MEDLINE

C.   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FANG et al. Dog Mastocytoma Cells Secrete a 92-kD Gelatinase Activated Extracellularly by Mast Cell Chymase. J.Clin.Invest. 01 April 1996, Vol. 97, No. 7,pages 1589-1596, see entire document.	1-32
Y	US 5,585,356 A (LIOTTA ET AL) 17 December 1996(12/17/96), see entire document.	1-32

☐ Further documents are listed in the continuation of Box C.   ☐   See patent family annex.

•	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
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"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 JULY 1999	31 AUG 1999

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